MATERIALS AND METHODS FOR DETECTION OF PATHOGENIC *GUIGNARDIA CITRICARPA*

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CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority under 35 U.S.C. §119(e) to US Provisional Application 60,177,013 filed January 19, 2000, the entire disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the fields of molecular biology and the detection of detrimental fungal species. More specifically, the invention provides materials and methods which facilitate the differentiation of pathogenic *Guignardia citricarpa* species from non-pathogenic *Guignardia* species.

BACKGROUND OF THE INVENTION

Black Spot of *Citrus* is a cosmetic disease on fruit of most of the commercial *Citrus* cultivars, including oranges, lemons, limes, and grapefruit. It begins with tiny black pustules on the surface of the developing fruit and, at room temperature, progresses to form highly developed, even all-encompassing black surface lesions. Although the disease originated in Africa, it has now spread to many areas of the world where *Citrus* are grown, including Brazil, Argentina, and China. The disease has not been reported in the United States and continued exclusion of the causative pathogen is a major concern of the USDA Plant Quarantine Service. The European Community has also adopted stringent measures to exclude spotted fruit from its borders.

The causative agent of *Citrus* Blackspot, *Guignardia citricarpa* (asexual state = *Phyllosticta citricarpa*) is a representative of a large and ubiquitous group of leaf-spotting *Ascomycetes*. Over 600 species of *Phyllosticta* have been reported in the United States, although many of these species descriptions are inadequate, and the actual number probably lies between 100-200. *Phyllosticta* spp. commonly form latent infections in many non-host plants, and are among the most commonly isolated asymptomatic fungal endophytes in leaves. For many years considerable confusion surrounded work on the taxonomic identity of *Guignardia citricarpa* and its pathogenicity on *Citrus* fruit. This confusion was finally resolved when it was discovered that there are actually two species of *Guignardia* on *Citrus*, and that one fruit, e.g., a single orange, may be infected simultaneously with both species. One species, the true *G. citricarpa*, is pathogenic, causes black spots on fruit, and is believed to be confined to the genus *Citrus*. The second species is non-pathogenic, forms insignificant lesions on fruit, and is widely dispersed on a huge number of unrelated host plants. The two fungal species are morphologically almost indistinguishable.

It would be highly advantageous to the *Citrus* fruit industry if means were available to differentiate between these two species of *Guignardia*.

SUMMARY OF THE INVENTION

Materials and methods are provided which facilitate the differentiation between pathogenic and non-pathogenic species of *Guignardia*.

In a preferred embodiment of the invention, oligonucleotides are provided which specifically and selectively hybridize with pathogenic vs. non-pathogenic

species of *Guignardia*. In one embodiment, the oligonucleotides are used for priming a DNA amplification of a target DNA sequence associated with pathogenic *Guignardia* is provided. Suitable oligonucleotide forward primers may be selected from the group consisting of those having the sequences AAAAAGCCGCCCGACCTACCT (SEQ ID NO: 1) and TAAAAAAGCCGCCCGACCTAC (SEQ ID NO: 8). Also provided is an oligonucleotide primer for forward priming a DNA amplification of a target DNA sequence associated with non-pathogenic *Guignardia citricarpa*. Suitable oligonucleotides may be selected from the group consisting of GCTACAACGCCGAAATGACCTT (SEQ ID NO: 2), GCCGTCGCCCAGCACTC (SEQ ID NO: 3), and GCTACAACGCCGAAATGACC (SEQ ID NO: 9). The following reverse primers are suitable for use in the amplification reactions disclosed above; SEQ ID NO: 6, SEQ ID NO: 10, and SEQ ID NO: 11.

In yet another embodiment of the invention, a method is disclosed for differentiating between pathogenic and non-pathogenic species of *Guignardia* in *Citrus* isolates. The method comprises i) obtaining a DNA sample from a *Citrus* fruit suspected of being infected with *Guignardia*; ii) contacting the fungal DNA with detectably labeled nucleic acid probes which bind pathogenic and non-pathogenic species of *Guignardia* selectively. Binding of a target fungal sequence being detectable by measuring the amount of detectably labeled probe bound. An alternative method comprises, i) obtaining a DNA sample from a *Citrus* fruit suspected of being infected with *Guignardia* and immobilizing the DNA on a solid support; iii) contacting the immobilized sample DNA with a detectably labeled oligonucleotide probe having a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 8, and SEQ ID NO: 9; and iv) assessing the solid support for

hybridization of the probe to the immobilized DNA. Samples demonstrating hybridization with SEQ ID NOS: 1 or 8 are associated with pathogenic *Guignardia* infection. Samples demonstrating hybridization with SEQ ID NOS: 2, 3, or 9 are associated with the non-pathogenic *Guignardia* species.

Also within the scope of the present invention, are materials and methods for selectively amplifying nucleic acid sequences which correspond to pathogenic *Guignardia citricarpa*. For amplification of DNA associated with pathogenicity, the fungal isolate is cultured for a suitable time period. The hyphae are harvested and lysed, and the DNA is isolated from the lysate. Alternatively, DNA may be directly isolated from the black spot lesion. The isolated DNA is then incubated with a primer pair which comprises forward primers SEQ ID NO: 1 or SEQ ID NO: 8 and a reverse primer selected from the group consisting of SEQ ID NO: 6 and TGCAATTCACATTACTTATCGC (SEQ ID NO: 11) under conditions suitable for PCR amplification. Preferably the primer pairs are SEQ ID NOS: 8 and 11. Also provided are materials and methods for selectively amplifying nucleic acid sequences which correspond to the non-pathogenic *Guignardia* species. For amplification of DNA associated with such non-pathogenic isolates, a suitable forward primer comprises SEQ ID NO: 2 or SEQ ID NO: 9 and a reverse primer selected from the group consisting of SEQ ID NO: 6, ATGCCAGAACCAAGAGATCC (SEQ ID NO: 10), and SEQ ID NO: 11. Preferably, the primer pairs are SEQ ID NOS: 9 and 10.

In a particularly preferred embodiment, kits for practicing the methods of the invention are provided. Components of such a kit include a pathogen-specific oligonucleotide probe, optionally immobilized on solid matrix, including means for amplifying the target fungal

nucleic acid in a test sample, the nucleic acid encoding all or part of the rDNA gene and comprising a sequence of SEQ ID NO: 4. The amplifying means within the kit comprises a primer pair of oligonucleotides having the sequence of, for example, SEQ ID NO: 1 and SEQ ID NO: 6 or SEQ ID NO: 8 and SEQ ID NO: 11; and reagents necessary to perform PCR. The kit may also comprise a non-pathogen-specific oligonucleotide probe immobilized on solid matrix for amplifying a sequence having the sequence of SEQ ID NO: 5. A primer pair of oligonucleotides suitable for amplifying DNA from non-pathogenic *Guignardia* have the sequence of, for example, SEQ ID NO: 2 and SEQ ID NO: 6 respectively. Solid matrix supports suitable for such hybridizations and polymerizations are disclosed in US patent 5,932,711, the entire disclosure of which is incorporated by reference herein. Preferably the primer pairs for amplifying pathogenic *Guignardia* are SEQ ID NOS: 8 and 11 and the primer pairs for amplifying non-pathogenic *Guignardia* are SEQ ID NOS: 9 and 10.

Various terms relating to the biological molecules and fungi of the present invention are used throughout the specification and claims.

With reference to nucleic acids used in the invention, the term "isolated nucleic acid" is sometimes employed. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule. An isolated

nucleic acid molecule inserted into a vector is also sometimes referred to herein as a "recombinant" nucleic acid molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein or compound of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In

particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single stranded nucleic acid molecules of varying complementarity are well known in the art.

For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., 1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The phrase "consisting essentially of" when referring to a particular nucleotide acid means a sequence having the properties of a given SEQ ID No:. For example, when used in reference to a nucleic acid

sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and functional characteristics of the sequence. Accordingly, the probes and primers of the respective SEQ ID NOS: of the invention may be modified slightly, i.e., via the addition of about 5 - 25 nucleotides, or by the substitution of 1 to 5 nucleotides in the recited sequence. Despite such modifications, however, the sequences perform the function recited in the assay or kit.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although the number of nucleotides may vary. The probes herein are selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively,

non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The phrase "solid matrix" as used herein includes, without limitation, filter paper, multiwell dishes, microchips, derivatized magnetic particles and the like.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 nucleotides in length, although this number may vary. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact

complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents an rDNA sequence isolate associated with pathogenic *Guignardia* strains (SEQ ID NO: 4).

Figure 2 an rDNA sequence isolate associated with non-pathogenic *Citrus* isolates (SEQ ID NO: 5).

DETAILED DESCRIPTION OF THE INVENTION

Each of the cells of all life forms, except viruses, contain ribosomes and therefore ribosomal RNA. A ribosome contains four separate single-stranded RNA molecules, namely, a large molecule (26S), a medium sized molecule (18S), and two small molecules (5.8S and 5S). The two larger R-RNA molecules vary in size in different organisms.

Ribosomal RNA is a direct gene product and is coded for by the R-RNA gene. This DNA sequence is used as a template to synthesize R-RNA molecules. A separate gene exists for each of the ribosomal RNA subunits. Multiple R-RNA genes exist in most organisms and many higher

organisms contain both nuclear and mitochondrial R-RNA genes. Plants and certain other organisms contain nuclear, mitochondrial and chloroplast R-RNA genes. The R-RNA gene and gene product have been well characterized in certain species. Hybridization of R-RNA and ribosomal genes in genetic analysis and evolution and taxonomic classification of organisms and ribosomal gene sequences has been described. Genetic analysis may include, for example, the determination of the numbers of ribosomal RNA genes in various organisms; the determination of the similarity between the multiple ribosomal RNA genes which are present in cells; determination of the rate and extent of synthesis of R-RNA in cells and the factors which control them.

In accordance with the present invention, molecular probes have been developed which distinguish between pathogenic and non-pathogenic *Guignardia citricarpa* species unambiguously, based on rDNA sequence differences. These probes may be used to advantage in methods routinely practiced in the laboratory setting.

Phylogenetically informative segments of ribosomal DNA from a large variety of *Phyllosticta* isolates have been sequenced in accordance with the present invention. The present invention provides a specific nucleic acid sequences isolated from *Citrus* fungus which differentiate *Citrus* fungal isolates which cause Black Spot symptoms from those which do not. The particular sequences studied to date include the internal transcribed spacer (ITS) 1 and ITS 2 segments of the *Guignardia* ribosomal DNA genes. From this work, it is clear that the pathogenic and non-pathogenic *Guignardia citricarpa* are quite different species with very different ITS sequences. Accordingly, species-specific PCR primers have been designed for use in an unequivocal and inexpensive method

to differentiate between pathogenic and non-pathogenic *Guignardia* species.

I. Preparation of Nucleic Acid Molecules, Probes and Primers which Differentiate Between Pathogenic and Non-Pathogenic *Guignardia* Species

A. Nucleic Acid Molecules

Nucleic acid molecules encoding the differentiating oligonucleotides of the invention may be prepared by two general methods: (1) synthesis from appropriate nucleotide triphosphates, or (2) isolation from biological sources. Both methods utilize protocols well known in the art. The availability of nucleotide sequence information, such as a DNA having the sequence of SEQ ID NOS: disclosed herein enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be used directly or purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

Figures 1 and 2 show the sequence differences between the ITS regions of pathogenic and non-pathogenic species of *Guignardia*. Accordingly, specific probes for identifying such sequences may be between 15 and 40 nucleotides in length. For probes longer than those described above, the additional contiguous nucleotides are provided within SEQ ID NOS: 4 and 5.

In accordance with the present invention, nucleic acids having the appropriate level of sequence homology with the sequences provided herein may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al.,

Molecular Cloning, Cold Spring Harbor Laboratory (1989), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

The nucleic acid molecules of the invention include genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the sequences provided herein. Also contemplated in the scope of the present invention are oligonucleotide probes which specifically hybridize with the DNA from pathogenic species of *Guignardia* while not hybridizing with DNA from the non-pathogenic species sequence under high stringency conditions. Primers capable of specifically amplifying the ITS segments of *Guignardia* rDNA encoding nucleic acids described herein are also contemplated to be within the scope of the present invention. As mentioned previously, such oligonucleotides are useful as primers for detecting, isolating and amplifying sequences associated with pathogenic *Guignardia*.

It will be appreciated by persons skilled in the art that variants (e.g., allelic variants) of the ITS sequences exist in the fungus population, and must be taken into account when designing and/or utilizing

oligonucleotides of the invention. Accordingly, it is within the scope of the present invention to encompass such variants, with respect to the ITS sequences disclosed herein or the oligonucleotides targeted to specific locations on the respective genes or RNA transcripts. Accordingly, the term "natural allelic variants" is used herein to refer to various specific nucleotide sequences of the invention and variants thereof that would occur in a population. The occurrence of genetic polymorphisms which give rise to minor base changes in a DNA molecule are known to those of ordinary skill in the art. Additionally, the term "substantially complementary" refers to oligonucleotide sequences that may not be perfectly matched to a target sequence, but such mismatches do not materially affect the ability of the oligonucleotide to hybridize with its target sequence under the conditions described.

II. DETECTION OF BLACK SPOT DISEASE-ASSOCIATED *Guignardia* SEQUENCES AND DIAGNOSTIC SCREENING ASSAYS THEREFORE

Currently, the most direct method for sequence analysis is DNA sequencing; however, it is also the most labor intensive and expensive method. Additionally, it is usually not practical to sequence all potentially relevant regions of every experimental sample. Other exemplary approaches for detecting the presence of pathogenic vs. non-pathogenic species of *Guignardia* based on nucleic acid differences include, for example:

a) comparing the sequence of nucleic acid in the sample with nucleic acid sequences from the non-pathogenic and pathogenic species of *Guignardia* to determine which species is present on the fruit; or

b) using DNA restriction mapping to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the suspect fungus as compared with the restriction pattern obtained from pathogenic and non-pathogenic species of *Guignardia*; or

c) using a specific binding member capable of binding to either the non-pathogenic or pathogenic nucleic acid sequence, the specific binding member comprising nucleic acids which distinguish between the two species based on hybridization specificities, or alternatively comprising an antibody or antibody fragment with specificity for a pathogenic or non-pathogenic *Guignardia* nucleic acid sequence, the specific binding member being labeled so that binding of the specific binding member to its binding partner is detectable; or

d) *in situ* hybridization between lesion DNA from permeabilized tissue sections and the use of fluorescent molecular probes specific for pathogenic or non-pathogenic *Guignardia* species; or

e) using PCR involving one or more primers based on non-pathogenic or pathogenic *Guignardia* gene sequences to screen for the presence of the pathogenic species in a sample.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which under normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands and receptors and complementary nucleotide sequences. The skilled person is aware of many other examples and do not need to be listed here. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise part of a large molecule. In embodiments in which the specific binding

pair consists of nucleic acid sequences, such sequences will be of a length to hybridize to each other under conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

In most embodiments for screening for the presence of pathogenic *Guignardia*, the nucleic acid associated with the pathogenic phenotype in the sample will initially be amplified, e.g. using PCR, to increase the amount of the analyte as compared to other sequences present in the sample. This allows the target sequences to be detected with a high degree of sensitivity if they are present in the sample. This initial step may be avoided by using highly sensitive array techniques that are becoming increasingly important in the art.

The identification of the nucleic acids associated with *Guignardia* pathogenicity paves the way for aspects of the present invention which provides the use of materials and methods, such as are disclosed and discussed above, for establishing the presence or absence in a test sample of the pathogenic species of the *Guignardia* fungus.

The invention also allows for planning of appropriate quarantine and/or prophylactic measures and permits stream-lining of analysis of infected *Citrus* fruits.

The following example is provided to illustrate an embodiment of the invention. It is not intended to limit the invention in any way. All temperatures are reported in centigrade degrees (°C) unless otherwise noted.

EXAMPLE I

Isolation and Characterization of Pathogenic *Guignardia* ITS Sequences

The following experimental protocols are provided to facilitate the practice of the present invention.

Suspect *Guignardia* isolates were grown on 60 mm plastic petri plates containing a modified PDA medium: 20 g crude agar, 24 g Difco Potato Dextrose Broth, 1g yeast extract, 1g malt extract, 940 mL Distilled Water. After the medium had been autoclaved and cooled to approximately 65º C the following liquid supplements were added before pouring the plates: 20 mL Antibiotic Stock to inhibit bacterial growth (5 mg/mL penicillin, 5 mg/mL streptomycin, 1.5 mg/mL chloramphenicol). Optionally, 10 mL Tricyclazole stock to inhibit melanin polymerization in cell walls (100 µg/mL) may be included. Additionally, 20 mL Ediphenphos stock to inhibit chitin formation in cell walls (2µL/mL) may optionally be included. Cultures were optionally planted on inert membranes such as Nuclepore filters to facilitate removal of mycelium for extraction. Fungal isolates may alternatively be cultured in liquid culture, preferably in 5% carrot juice.

After three to five days of culture, 2-10 mg fresh weight of hyphae were scraped from the plate and placed into a 600 µL Eppendorf vial with 100 µL Epicentre Tfl (x1) buffer. The tissue was then subjected to 3 cycles of freezing (liquid nitrogen, 2 min) and heating (90º, 5 min). Next, 5µL of extract were withdrawn from each sample and mixed with an equal volume of the following PCR reaction mix (total 100 µL) to amplify target rDNA: 4.0M Betaine (Acros Chemical Co.) - 40µL; Magnesium chloride, 25mM - 22µL; dNTP's mixed, 2.5mM, 16µL; Tfl buffer (x20) - 10µL; Bovine serum albumin, 10mg/mL, - 5µL; Distilled water - 5.4 µL; Promega Taq polymerase,

1U/μL, 2μL; reverse primer ITS 4(5' TCCTCCGCTTATTGATATGC 3'; SEQ ID NO: 12), 10mM, 0.8μL; and forward primer ITS 5 (5' GGAAGTAAAAGTCGTAACAAGG 3'; SEQ ID NO: 13), 10mM, 0.8μL. Other reagents are available for performing PCR including Epicentre MasterAmp which may be used in place of Betaine and Epicentre Tfl DNA polymerase which may be used in place of Promega Taq polymerase.

Each 10μL reaction mix was loaded into a 10μL capacity glass capillary tube; the ends were sealed and loaded into a RapidCycler (Idaho Technology) PCR machine and run with the following program: Hold: 94º, 15 sec. Cycle: Denature 94º, 1 sec; Anneal 55º, 3 sec; Elongation, 72º, 15 sec - slope 2.0, 39 cycles. Hold: 72º, 1 minute. The contents of the capillary tube were loaded into a PCR vial, and a second PCR reaction was prepared to amplify the pathogenic *Guignardia* sequence (if present) as follows. Mix A: 52 μL distilled water; 10 μL Tfl buffer (x20); 2μμL Epicentre Tfl DNA polymerase. Mix B. 40μL Tfl MasterAmp (X10); 22μL magnesium chloride; 17 μL distilled water; 16μL dNTP's; 5μL BSA. To a 200 μL PCR tube add 5μL Mix B, 3.2 μL Mix A, 0.8 μL CIT 1 ITS forward primer (5' AAAAAGCCGCCCGACCTACCT 3'; SEQ ID NO: 1), 0.8 μL reverse primer ITS 2 (5' GCTGCGTTCTTCATCGATGC 3' (SEQ ID NO: 6), and 0.2-0.3 μL template DNA taken directly from the previous PCR reaction mix. The sample is then run on a Rapid Cycler using the same program as the initial PCR reaction. Alternatively, SEQ ID NOS: 8 and 6 may be used as the forward and reverse primers respectively.

Following completion of the thermocycling program, a 2% agarose gel (electrophoresis grade) prepared with a narrow comb was loaded with 3-5 μL of sample in 4-5 μL of loading buffer (total volume 7-10μL) and run in TBE buffer at 80 V for 30 minutes. Following electrophoresis the entire gel was stained with SYBAR Gold (Molecular

Probes), 1:10,000 dilution for 15 minutes. The gel was placed on a Clare Dark Reader. A positive reaction was evident as a bright band on the order of 150 bp. If the band was present, it was concluded that the *Guignardia* species under investigation is pathogenic. To ensure this observation, the primers specific for the non-pathogenic species were substituted for CIT1-ITS1. These primer pairs specifically amplify DNA from the non-pathogenic *Guignardia* species and have the following sequences. CIT3- ITS1 forward primer (5' GCTACAACGCCGAAATGACCTT 3'; SEQ ID NO: 2) and ITS2 reverse primer (5' GCTGCGTTCTTCATCGATGC 3'; SEQ ID NO: 6) or ITS3 forward primer (5' GCATCGATGAAGAACGCAGC 3'; SEQ ID NO: 7) and CIT3-ITS2R reverse primer (5'GCCGTCGCCCAGCACTC 3'; SEQ ID NO: 3). Figure 1 shows the rDNA ITS sequence associated with pathogenic *Guignardia* , SEQ ID NO: 4. Figure 2 shows the rDNA ITS sequence associated with non-pathogenic *Guignardia* , SEQ ID NO: 5.

EXAMPLE II

Alternative Isolation of *Guignardia* DNA

A sample of *Guignardia* from a *Citrus* fruit was cultured by inoculation of the sample into a solution of 5% aqueous carrot juice. Following a suitable incubation period (typically between 72 hours and 1 week at 25°C), mycelia were removed from the culture medium and rinsed with distilled water. The rinsed mycelia were added to PCR buffer and heated at 90°C for 10 minutes to release fungal DNA. An aliquot (1/10 of the total volume) of the heated fungal DNA-containing PCR buffer was amplified as described in Example I. The amplified sample was separated by gel electrophoresis, stained, and read according to the methods described in Example I.

A further refinement of this method may be achieved utilizing sequence specific fluorescent probes which bind to the middle of the sequence during PCR. These are known as "Molecular Beacons", and would confer greater sensitivity and specificity on the procedure described above. Additionally, the fluorescence could be directly detected in the PCR mix thereby obviating the need for detecting bands on agarose gels.

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SEQUENCES UTILIZED IN THE PRESENT INVENTION

SEQ ID NOS: 1 and 8 - Forward primers for amplification of pathogenic *Guignardia* species

SEQ ID NOS: 2, 3, 7 and 9 - Forward primers for amplification of non-pathogenic *Guignardia* species.

SEQ ID NO: 4 - rRNA sequence from pathogenic *Guignardia* species

SEQ ID NO: 5 - rRNA sequence from non-pathogenic *Guignardia* species

SEQ ID NOS: 6, 10 and 11 - Reverse primers for amplification of both pathogenic and non-pathogenic *Guignardia* species

SEQ ID NOS: 12 and 13 - Forward and reverse primers for the amplification of internal transcribed spacer regions from *Guignardia*.

While a preferred embodiment of the present invention has been described and specifically exemplified above, it is not intended that the invention be limited to such an embodiment. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.